

**VERIFICATION OF TRANSLATION**

**JAPANESE PATENT APPLICATION NO. 2004-122898 FOR "METHOD OF CULTURING  
ADHESIVE CELLS"**

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Toranomon 1-chome, Minato-ku, Tokyo 105-0001 Japan hereby declare as follows:-

1. I am a translator and am familiar with both the English and Japanese languages.
2. I am the translator of the document(s) attached hereto and certify that the following is a true translation of priority document of Japanese Patent Application No. 2004-122898 filed on April 19, 2004 to the best of my knowledge and belief.

Dated this 28th day of November, 2011



Signature of Translator

Japanese patent application No. 2004-122898

## DESCRIPTION

### METHOD OF CULTURING ADHESIVE CELLS

#### TECHNICAL FIELD

[0001]

The present invention relates to a method of culturing adhesive cells, particularly to a method of producing a virus on an industrial scale by the cell culture.

#### BACKGROUND ART

[0002]

Recently, a risk of zoonotic infectious disease such as BSE (Bovine Spongiform Encephalopathy) infection and Avian Influenza virus infection, has started to be a concern. Moreover, products using ingredients or materials primarily originated from animals (such as vaccines, blood preparations, cell culture/genetic recombinant preparations, and cellular tissue medical products) have problems of a high risk where an infectious agent is mixed thereinto, and an undeniable likelihood of containing an unknown infectious agent, as well as a limitation in the inactivation treatment of infectious agents, and the like. As a countermeasure against such problems, legal countermeasures for safety have been enhanced, such as newly providing a framework for “bio-originated products” by the amendment of Japanese Pharmaceutical Law in 2003, and there has become a desire for the development of medical products free from animal-origin components.

[0003]

Meantime, as for industrial-scale methods of producing biological drug products such as a vaccine and a monoclonal antibody, those by the cell culture have been considered to be useful since a large amount of the vaccines and the monoclonal antibodies that are of stable quality, can be obtained. Animal cells used in these methods are often adhesive, and these adhesive cells adhere to a solid surface and proliferate. For example, a culturing method using a support such as a microcarrier is known as a method of culturing a large amount of the adhesive cells.

[0004]

For example, Patent Document 1 discloses a method of producing a virus on an industrial scale using adhesive cells comprising steps of: proliferating Vero cells using both Vero cells and a microcarrier of Cytodex1 (registered trademark); and then replacing a medium with another medium containing 5 g/L of animal-origin protein to produce a Japanese encephalitis virus. Non Patent Document 1 discloses a method of proliferating cells using Vero cells, a microcarrier of Cytodex1 (registered trademark) and a serum-containing medium originated from animals, and then producing an inactivated poliovirus. Furthermore, Patent Document 2 discloses a method of proliferating cells using Vero cells, a microcarrier of Cytodex1 (registered trademark) and a serum-containing medium, and then producing a Japanese encephalitis virus using a serum-free medium. However, such methods using a serum-containing medium originated from animals have problems of an undeniable likelihood of containing an unknown infectious agent and the like as described above.

[0005]

From these backgrounds, many attempts have been made for obtaining a culture condition not requiring a serum, such as bringing serum-free mediums free from animal-origin components into the market by respective medium manufacturers. However, in a culture method using a serum-free medium and a microcarrier where the surface of the carrier is electrically charged at an appropriate amount so as to adhere cells, as a support for culturing adhesive cells, there has been a problem in that the attachment rate to the microcarrier is reduced, making it difficult to efficiently culture a large amount of cells. As a result, conventionally, under such a culture condition, there has been used a carrier containing animal-origin components such as a microcarrier coated with denatured pig collagen, as described for example in Non Patent Document 2.

[0006]

Moreover, there is disclosed in Patent Document 3, a bead for culturing animal cells which has a high cell-adhering property and a high cell-proliferating property, and, even in a serum-free culture medium, gives a cell-adhering property and a cell-proliferating property which are equivalent or more to those in serum-containing medium. However, although Patent Document 3 shows that cells can be efficiently cultured under a serum-free condition in a cell culture, there is no disclosure of a condition for culturing a virus.

[0007]

Moreover, there is disclosed in Patent Document 4, a method of producing a virus including steps of: obtaining a vertebrate cell culture such as Vero cells; proliferating the cells

only in a protein-free medium (free from serum or non-serum protein); infecting this culture with a virus; incubating the virus-infected cell culture; proliferating the virus in the medium; and producing the virus-containing medium. Furthermore, there is disclosed a usage of a protease originated from a prokaryote supply source as a substance which enhances the virus activity. Patent Document 4 describes that, according to this method, the obtained virus do not contain various impurity compounds originated from a human or animal supply source, nor a protein serving as a pathogenic substance. However, in an Example a trypsin which is an animal-origin component is used as a substance which enhances the virus activity.

[0008]

Furthermore, there is disclosed in Patent Document 5, a method of producing a virus infected insect cell not using a naturally-originated protein but using a cell-adhesive support having a high cell-adhering property. This production method is a method of producing a virus infected insect cell, comprising steps of; using a cell-adhesive artificial peptide and/or a cell-adhesive auxiliary artificial peptide to adhere a poikilothermic animal-origin cell and a substrate, and using this cell-adhered substrate for culturing cells. Patent Document 5 describes that, by not using a naturally-originated protein for a substrate, there is no risk of containing an infectious substance such as a human-infective virus, and the safety is high. However, there is a disclosure of using a medium containing serum originated from animals as a medium for cultivating a virus infected insect cell.

[0009]

On the other hand, for the subculture of cells in Patent Document 2 and Non Patent Document 2, an animal-origin protease (such as pig-origin trypsin) is used as a cell dispersing agent.

Patent Document 1: Japanese Unexamined Patent Publication No. H11-510151

Patent Document 2: Japanese Patent Publication No. 2000-83657

Patent Document 3: Japanese Unexamined Patent Publication No. 2003-189848

Patent Document 4: Japanese Patent Publication No. 3158157

Patent Document 5: Japanese Unexamined Patent Publication No. 2003-210166

Non Patent Document 1: Montangnon B. J., Fanget B., Nicolas A. J. Develop. Biol. Standard 47, 55-56 (1981)

Non Patent Document 2: Otfried Kisner et al. "Development of a Novel Mammalian Cell (Vero) Derived influenza Vaccine" Poster Presented at: Options for Control of Influenza V, Okinawa, Japan, October 7-11, 2003

## DISCLOSURE OF THE INVENTION

### Problems to be solved by the Invention

[0010]

Therefore, an object of the present invention is to provide a safe and large-scale method of culturing adhesive cells and of producing a virus in a culturing system free from animal-origin components in the whole process from culturing adhesive cells to producing the virus on an industrial scale by the cell culture.

### Means for solving the Problem

[0011]

The above problems have been considered and earnestly studied, resulting in a finding of a method of culturing a large amount of adhesive cells which are safe and of stable quality by using a culture material which is free from animal-origin components, and thus conceiving the present invention.

[0012]

A first aspect of the present invention is a method of culturing adhesive cells comprising: adhering adhesive cells to a support free from animal-origin components; culturing the adhesive cells in a medium free from animal-origin components; and subculturing the cultured adhesive cells using a cell dispersing agent free from animal-origin components.

[0013]

A second aspect of the present invention is the method according to the first aspect, wherein said support is a microcarrier.

[0014]

A third aspect of the present invention is the method according to the second aspect, wherein said microcarrier is coated with a recombinant adhesive factor.

[0015]

A fourth aspect of the present invention is the method according to the first aspect, wherein said cell dispersing agent is a recombinant enzyme.

[0016]

A fifth aspect of the present invention is the method according to the first aspect, wherein said adhesive cells are of homoiothermic animal-origin.

[0017]

A sixth aspect of the present invention is a method of producing a virus comprising: adhering adhesive cells to a support free from animal-origin components; culturing the adhesive cells in a medium free from animal-origin components; subculturing the cultured adhesive cells using a cell dispersing agent free from animal-origin components; inoculating a virus in the cells obtained by culturing the adhesive cells; and then recovering the virus.

#### Effects of the Invention

[0018]

According to the method of culturing adhesive cells as set forth in the first aspect of the present invention, since culture materials are free from animal-origin components, a large amount of adhesive cells which are safe and of stable quality, can be cultured.

[0019]

According to the method of culturing adhesive cells as set forth in the second aspect of the present invention, a large amount of adhesive cells can be cultured.

[0020]

According to the method of culturing adhesive cells as set forth in the third aspect of the present invention, since culture materials are free from animal-origin components, a large amount of adhesive cells which are safe and of stable quality, can be cultured.

[0021]

According to the method of culturing adhesive cells as set forth in the fourth aspect of the present invention, since culture materials are free from animal-origin components, a large amount of adhesive cells which are safe and of stable quality, can be cultured.

[0022]

According to the method of culturing adhesive cells as set forth in the fifth aspect of the present invention, since culture materials are free from animal-origin components, a large amount of adhesive cells which are safe and of stable quality, can be cultured.

[0023]

According to the method of culturing adhesive cells as set forth in the sixth aspect of the present invention, since culture materials are free from animal-origin components, a large amount of adhesive cells which are safe and of stable quality, can be cultured.

#### BEST MODE FOR CARRYING OUT THE INVENTION

[0024]

Hereunder is a detailed description of embodiments of the present invention.

[0025]

In the present invention, “free from animal-origin components” means free from components originated from homoiothermic animals, in particular, animals such as mammals (for example, human, cattle, pig, dog, rabbit, cat, and the like), birds, and fishes.

[0026]

Moreover, in the present invention, an adhesive cell is not specifically limited as long as it is a cell which grows by adhering onto a solid surface and is capable of culturing a virus. The adhesive cell is preferably of homoiothermic animal-origin. Examples of the adhesive cell originated from the homoiothermic animals include an epithelial cell (such as Vero, MDCK, CHO, HEK293, and COS), a tumor cell (such as Hela and VACO), an endothelial cell (such as HUVEC and DBAE), a leukocyte (such as HIT-T15), a fibroblast (such as WI38, BHK21, and SFME), a muscle cell (such as HL1 and C2C12), and a nerve/endocrine cell (such as ROC-1 and IMR-32).

[0027]

Moreover, examples of the virus to be inoculated in the adhesive cells in the present invention include Flaviviridae, Orthomyxoviridae, Adenoviridae, Herpesviridae, Picornaviridae, Paramyxoviridae, Togaviridae, Poxviridae and the like.

[0028]

Furthermore, the culture material (such as a medium, a cell dispersing agent, and a support for adhering adhesive cells) free from animal-origin components used in the present invention is free from animal-origin components, and therefore has advantages in that: it can minimize a likelihood of contamination by foreign substances; it is free from unknown infectious agents; and it has a low risk of being mixed with infectious agents, and thus there is no need for performing a treatment for deactivating the infectious agents. Hereunder is a detailed description of the culture material free from animal-origin components.

[0029]

The medium used for culturing an adhesive cell or producing a virus is not specifically limited as long as it is a medium free from a serum or a protein being animal-origin components. Examples thereof include VP-SFM (manufactured by Invitrogen) and the like. This VP-SFM is suitable for culturing a cell system, such as Vero, COS-7, MDCK, BHK-21, HEP-2 and the like,

and peculiarly for proliferating a virus and producing a recombinant protein and a monoclonal antibody.

[0030]

The support for adhering adhesive cells means a support to which adhesive cells can adhere to its surface so that the cells can grow thereon, the support being free from animal-origin components and having a high cell-adhering property. Examples of a substrate of the support include a microcarrier in the form of a bead made from dextran, acrylic resin, glass, polyethylene, polystyrene etc., and said microcarrier allows a large amount of cell, virus and the like to be cultured. The support also may be a microcarrier in which a substrate in the form of a bead is coated with e.g., a recombinant adhesive factor, and the most preferably coated with ProNectin (registered trademark), manufactured by Sanyo Chemical Industries,Ltd.), i.e., a protein synthesized using a recombinant E. coli, having an Arg Gly Asp sequence and a silk fibroin structure. The ProNectin (registered trademark) has greater cell-adhesive and proliferation activity than any prior adhesive factors (such as fibronectin), and thus it is capable of culturing cells at high density in a serum-free culture method.

[0031]

The cell dispersing agent means a cell dispersing agent free from animal-origin components used for subculture, and preferably a recombinant enzyme which is a high-purity fermented product or the like. This recombinant enzyme can be a replacement for an animal trypsin produced by microorganisms.

[0032]

The method of culturing adhesive cells of the present invention comprising: adhering adhesive cells to a support free from animal-origin components; culturing the adhesive cells in a medium free from animal-origin components; and subculturing the cultured adhesive cells using a cell dispersing agent free from animal-origin components. In the method of producing a virus of the present invention, a large amount of virus can be produced by: adhering adhesive cells to a support free from animal-origin components; culturing the adhesive cells in a medium free from animal-origin components; subculturing the cultured adhesive cells using a cell dispersing agent free from animal-origin components; and then inoculating a virus in the cells obtained by culturing the adhesive cells.

[0033]

According to the present invention, since culture materials free from animal-origin components are used in the process of cell culture and virus production, a large amount of

adhesive cells which are safe and of stable quality, can be cultured. Furthermore, it has advantages in that it can minimize the likelihood of contamination by foreign substances, it is free from unknown infectious agents

[0034]

The present invention is not limited to the above contents of description, and various modifications can be made within the scope of the present invention.

[0035]

Hereunder is a detailed description of the present invention, with reference to Examples and drawings. However, the present invention is not limited to these Examples.

Example 1

[0036]

<Cell culture and subculture of a Vero cell using a spinner flask>

A sample 1 comprising a Dulbecco's minimum essential medium (DMEM) medium + 5 volume % fetal bovine serum (FBS) and a microcarrier (Cytodex1 manufactured by Amersham); a sample 2 comprising a serum-free medium (VP-SFM manufactured by Invitrogen) free from animal-origin components and a microcarrier (Cytodex1 manufactured by Amersham); a sample 3 comprising a serum-free medium (VP-SFM manufactured by Invitrogen) free from animal-origin components and a microcarrier coated with a denatured pig collagen (Cytodex3 manufactured by Amersham); and a sample 4 comprising a serum-free medium (VP-SFM manufactured by Invitrogen) free from animal-origin components and a microcarrier in the form of a bead coated with a ProNectin (registered trademark) free from animal-origin components were each prepared and put into a different spinner flask. Next, the Vero cells during subculturing in the Dulbecco's minimum essential medium (DMEM) medium + 5 volume % fetal bovine serum (FBS) were disseminated in the each spinner flasks containing the above samples so that the cell density became  $2 \times 10^5$  cells/mL. Then, the culture solution containing the cells, the medium and the microcarrier was stirred, the cells adhered to the microcarrier, and then the cells were cultured for 24th days. The surface of the microcarrier of Cytodex1 is electrically charged at an appropriate amount so as to adhere to cells; the microcarrier of Cytodex3 is coated with a denatured pig collagen so as to adhere to cells; the microcarrier of ProNectin beads adhere to cells by the Arg Gly Asp sequence in the ProNectin. The subculture of the cells was performed every 8th days. From the 3rd day of the cell dissemination, 50 volume % of medium was exchanged every day. The cells were dispersed by a diluted solution having a cell dispersing agent free from animal-origin components, which was

used for subculture (rProtease (registered trademark) manufactured by Invitrogen) 5-fold diluted, under a condition of 37°C, and the filtrate was removed by centrifugation. Then, the required amount thereof was disseminated in a spinner flask that had been previously prepared, so as to perform the subculture operation. The change in the cell density of each sample with time was obtained by a general method such as hematometry and microscopy. A graph showing the cell density (cells/mL) on the y-axis and time on the x-axis, is shown in FIG. 1.

[0037]

From FIG. 1, it can be seen that the cell densities of the samples 2, 3 and 4 cultured in the serum-free mediums free from animal-origin components were over  $2 \times 10^6$  cells/mL when the subcultures of the cells were performed on the 8th and 16th days from the start of the cell culture. On the 24th day from the start of the cell culture, the cell densities of the samples 2 and 4 cultured in the serum-free mediums free from animal-origin components were  $3.0 \times 10^6$  cells/mL while the cell density of the sample 1 cultured in the DMEM medium + 5 volume % FBS was about  $1.6 \times 10^6$  cells/mL. Therefore, it was found that the method of culturing cells using culture materials free from animal-origin components can culture more cells than the prior method of culturing cells using culture materials containing animal-origin components, and culture cells without the possibility of contamination by foreign substances.

Example 2

[0038]

<Culture of a Japanese encephalitis virus by a spinner flask culture method>

Sampling was performed between the 2nd to 8th day of the JEV culture, and the number of cells was measured by measuring the number of nuclei after citric acid treatment. Regarding the index of JEV growth, (1) HA (Hemagglutination) value was measured according to the usual method, and (2) in ELISA (Enzyme-linked immunosorbent assay), antibody purification of anti-JEV antiserum was performed by means of protein A column and the reaction specificity was confirmed by means of Western Blotting, and then the purified antibodies were labeled with peroxidase, so as to construct a sandwich ELISA. Regarding the ELISA value, the autologous value from reference Japanese encephalitis vaccine Beijing strain Lot.197-P was added, and the purified inactive JEV solution was used as a reference antigen. A graph showing the change in the JEV production quantity with time by ELISA, is shown in FIG. 2. A graph showing the change in the JEV production quantity with time by HA, is shown in FIG. 3. Moreover, the highest values of ELISA and HA measurement values in each sample are shown in Table 1.

[0039]

Table 1

	Cell number at time of virus inoculation ( $\times 10^6$ cells/mL)	HA	ELISA (U/mL)
Sample 1	1.6	256	1949
Sample 2	3	128	1087
Sample 3	2.4	64	626
Sample 4	3	512	2367

[0040]

From FIGs. 2 and 3 and Table 1, it can be seen that the number of cells in the sample 1 cultured in a combination of the DMEM medium + 5 volume % FBS and a microcarrier (Cytodex1 manufactured by Amersham) was about  $1.6 \times 10^6$  cells/mL just before the virus dissemination and  $2.7 \times 10^6$  cells/mL on the 3rd day from the start of the virus culture, which reached a level similar to that in the samples 2 to 4 cultured in the serum-free mediums free from animal-origin components. The sample 4 cultured in a combination of the serum-free medium free from animal-origin components and the ProNectin beads free from animal-origin components showed higher values of both ELISA and HA (ELISA value: 2367 U/mL, HA value: 512 times) compared to the sample 1 cultured in a combination of the DMEM medium + 5 volume % FBS and a microcarrier (Cytodex1 manufactured by Amersham) (ELISA value: 1949 U/mL, HA value: 256 times). Furthermore, the sample 4 free from animal-origin components showed the highest values of both the number of cells and the virus production quantity in this Example. Therefore, it was found that the method of culturing cells using culture materials free from animal-origin components can culture more cells than the prior method of producing a virus using culture materials containing animal-origin components, and culture cells without the possibility of contamination by foreign substances.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0041]

FIG. 1 is a graph showing changes in cell density with time in Example 1.

FIG. 2 is a graph showing changes in virus production quantity by ELISA with time in Example 2.

FIG. 3 is a graph showing changes in virus production quantity by HA with time in Example 2.

Fig. 1

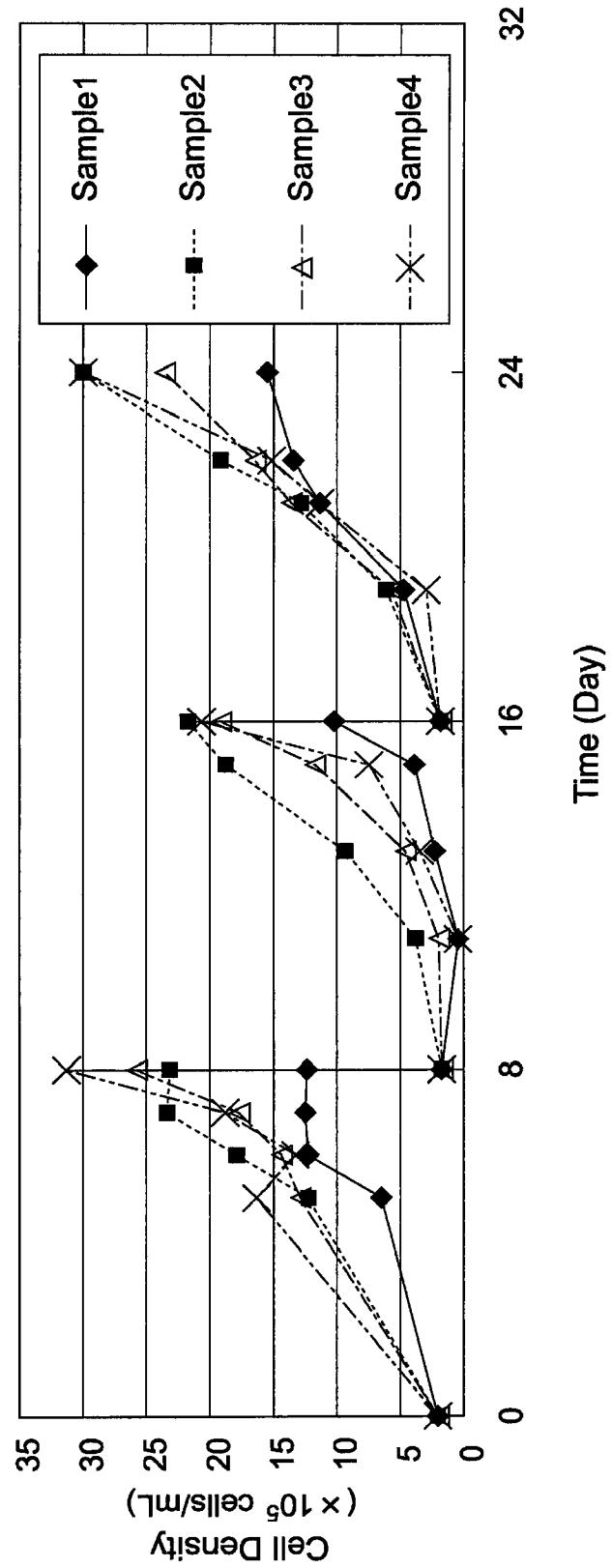


Fig. 2

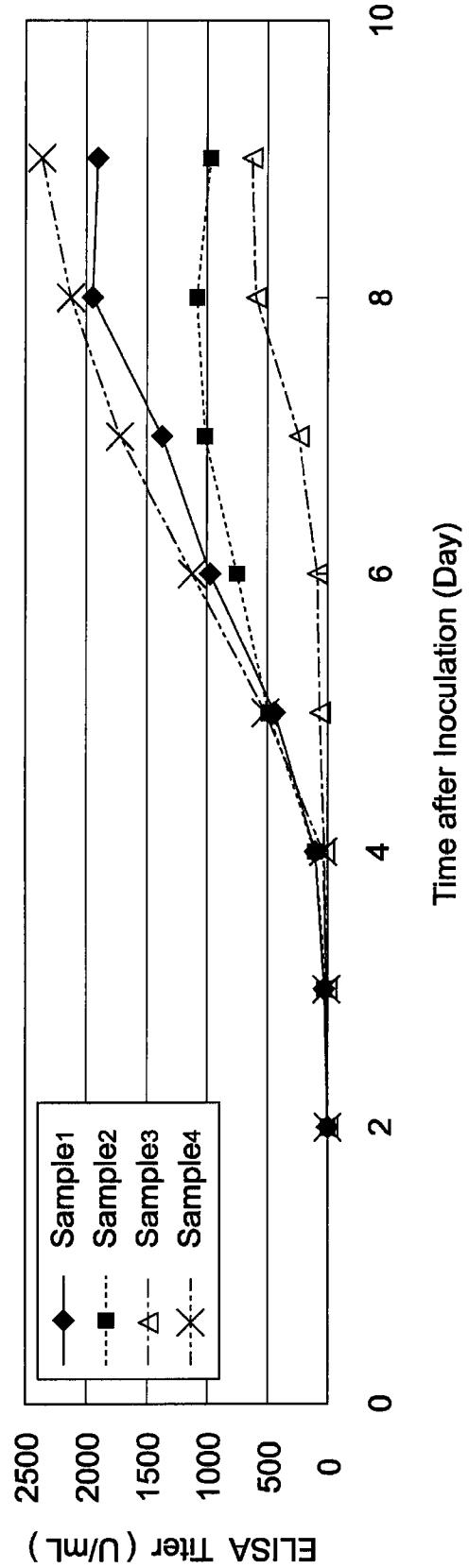
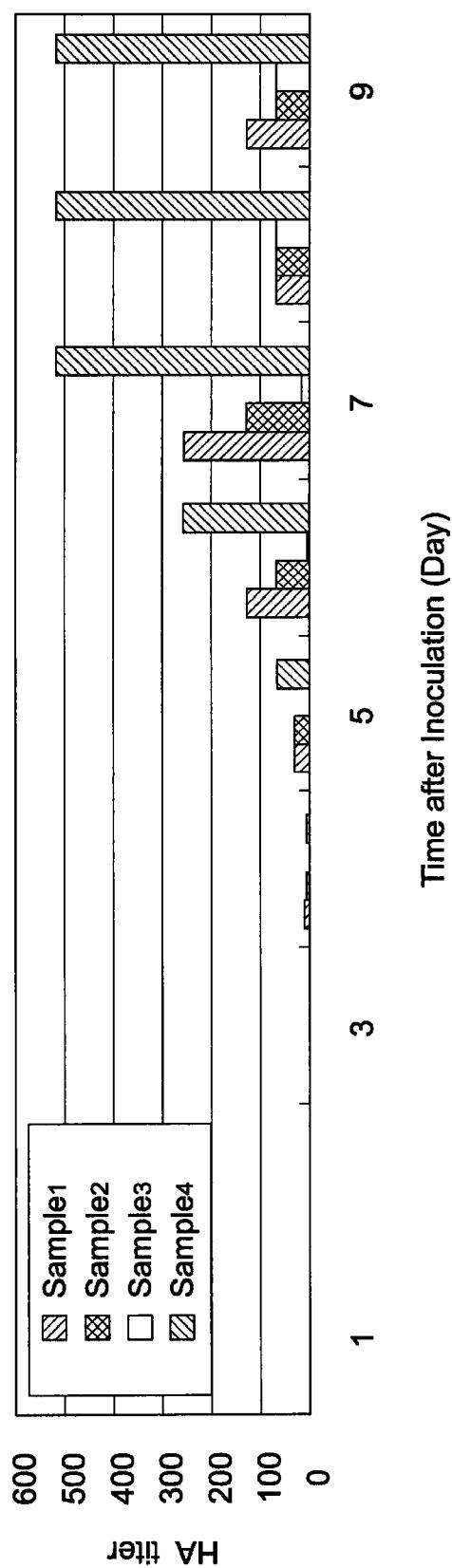


Fig. 3



## CLAIMS

- [1] A method of culturing adhesive cells comprising: adhering adhesive cells to a support which is free from animal-origin components; culturing the adhesive cells in a medium free from animal-origin components; and subculturing the cultured adhesive cells using a cell dispersing agent free from animal-origin components.
- [2] The method according to claim 1, wherein said support is a microcarrier.
- [3] The method according to claim 2, wherein said microcarrier is coated with a recombinant adhesive factor.
- [4] The method according to claim 1, wherein said cell dispersing agent is a recombinant enzyme.
- [5] The method according to claim 1, wherein said adhesive cells are of homoiothermic animal-origin.
- [6] A method of producing a virus comprising: adhering adhesive cells to a support free from animal-origin components; culturing the adhesive cells in a medium free from animal-origin components; subculturing the cultured adhesive cells using a cell dispersing agent free from animal-origin components; inoculating a virus in the cells obtained by culturing the adhesive cells; and then recovering the virus.

## ABSTRACT

[Problem to be solved] The present invention provides a safe and large-scale method of culturing adhesive cells and of producing a virus in a culturing system free from animal-origin components in the whole process from culturing adhesive cells to producing the virus on an industrial scale by the cell culture.

[Means for solving the problem] A large amount of adhesive cells which are safe and of stable quality can be cultured by using culture materials (such as a support for adhering adhesive cells, a medium used for culturing a cell and a cell dispersing agent used for the subculture) free from animal-origin components. Furthermore, it has advantages in that it can minimize the likelihood of contamination by foreign substances, it is free from unknown infectious agents, and thus there is no need of performing a treatment for deactivating the infectious agent.

[Representative drawing] None